OUTLINE OF THE PROPOSED WORK

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN PHARMACY

Submitted To

SWAMI RAMANAND TEERTH MARATHWADA UNIVERSITY, NANDED

Topic entitled

Validation of Ethanopharmacological Claims of Medicinal Plant and Bioactive Phytochemical for Antiangiogenic and Anticancer Activity

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2017

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Abbreviations

VEGF: Vascular Endothelial Factor, **NF-κB**: Nuclear factor-κB, **Bcl-2**: B-cell lymphoma 2, **Bcl-XL**: B-cell lymphoma-extra large, **STAT:** Signal transducer and activator of transcription, **TNF**: Tumor necrosis factors, **p53**: Tumor suppressor p53, **AP-1:** Activator protein 1, **PARP**: Poly ADP ribose polymerase, **Nrf2**: Nuclear factor erythroid 2, **Ig**: Immunoglobulin, **AMD**: Age-related macular degeneration, **TGF-α**: Transforming growth factor-α, **CAM**: Chorioallantoic membrane

1. INTRODUCTION

- The incidences of cancer are continuously increasing worldwide, affecting millions of people. Deaths due to cancer are projected to continuously increase and it has been estimated that there will be 11.5 million deaths in the year 2030 and 27 million new cancer cases and 17.5 million cancer deaths are projected to occur in the world by 2050. (Kadri et al., 2014).
- The world health organization estimated that 80% of the population in the world has regularly used traditional herbal medicines. In the United States and Europe, it is estimated that 25% of medical prescriptions contains plant ingredients (extracts, fractions, isolated phytoconsti) of ethnopharmacologically potential plant (Kadri et al., 2014).
- Francesca Grifo showed that 118 of the 150 most prescribed drugs in the United States were originally derived from living organisms, 74% of plant, 18% of mushrooms, 5% of bacteria and 3% of Bothrops (venomous snake). Nine of the top ten are based on natural products from plant (Kadri et al., 2014).
- Ancient civilizations of India, China and Egypt have been utilizing dietary herbs and spices for treating a variety of ailments apart from their common use as food flavoring and seasoning agents. Many bioactive compounds isolated from these herbs and spices have been reported to show preventive and therapeutic properties for cancer (Thanekar et al., 2016).
- Angiogenesis is the formation of a new network of vessels from preexisting vessels, a process that occurs via intussusceptions (budding). Tissue stimulation is requisite for inducion and increase the expression of pro-angiogenic factors such as Vascular

Endothelial Factor (VEGF) and Fibroblast Growth Factor for the occurrence of angiogenesis (Prado et al., 2016).

- Abnormal vessel growth and functions are hallmarks of cancer that contribute to the progression of solid malignancies. Because angiogenesis together with metastasis is essential for tumor growth beyond minimal size, and hence anti-angiogenesis has been considered as a potential therapeutic strategy for treating cancer. Among the numerous proangiogenic factors that have been identified as mediators of the angiogenic switch, vascular endothelial growth factor (VEGF) is one of the most powerful angiogenesis promoters, and plays a significant role in tumor neovascularization (Li et al., 2015).
- Plant based bioactive compounds such as curcumin, quercetin, resveratrol, glucosinolates, Ginger, Biocalein, Genistein, vinca alkaloids (vinblastine, vincristine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), podophyllotoxin and its derivations (topothecan, irinothecan), anthra cyclines (doxorubicin, daunorubicin, epirubicin, idarubicin), lectines, and xanthorrhizol exerts anticancer effects by several mechanisms.
- The principle anticancer mechanisms of phytochemicals include NF-κB (nuclear factor kappa-light chain) an enhancer of activated B cells, cyclin D1, apoptosis suppressor proteins such as Bcl-2 (B-cell lymphoma 2) and Bcl-XL (B-cell lymphoma-extra large), signal transducer and activator of transcription (STAT) proteins, TNF (tumor necrosis factors), p53 (tumor suppressor p53), AP-1 (activator protein 1) and PARP (polyADP ribose polymerase) (Thanekar et al., 2016).

2. LITERATURE REVIEW

- A continuous increase in the cancer incidences and mortality in developed and developing countries is a serious concern worldwide. **Cancer is the second major cause of mortality after the cardiovascular diseases**. Among the different types of cancer, the prevalence of lung cancer is more common in man and breast cancer in women. Several factors contribute to the development of cancer, which may be internal such as genetic alteration, mutation in the genetic material, abnormal hormonal condition and compromised immune system; and external causes may be the environmental factors including the modern lifestyle and specific occupation conditions (Loney et al., 2013; Singh et al. 2016; Coelho 2012).
- It is estimated that 60% of the world's population has a great dependence on plant for medication. In fact, more than half of the currently existing drugs are natural compounds or related and over 70% of anticancer drugs are derived from natural compounds (Seow et al., 2011).
- Literature survey also revealed that 60% people take herbs, vitamins, and herbal medicines to control cancer and associated complications. Use of medicinal plant can help to reduce toxic and side effects of chemotherapy and radiation treatment by reinforcing their anticancer effect. It is estimated that in the last decade, approximately 30.69 billion USD plant derived drugs has been estimated in the global market. Several phytochemicals produce strong anti-inflammatory and antioxidative actions mediated through Nrf2, NF-κB, and AP-1 signalling pathways. Similarly phytochemicals with anticancer potential can prevent cancer initiation, promotion and progression by interfering various intermittent mechanisms and pathways. Near about 12.4 billion USD

terpenes and steroids obtained from the plant were sale annually worldwide (Sak 2012; Safarzadeh et al. 2014; Pratheeshkumar et al. 2012).

- Traditional herbal medicines have long been recognized as a rich source for discovering new anticancer agents. Up to the present, many herbs and phytochemicals have been shown to have anti-angiogenic activities e.g. *Benincasa hispida, Withania somnifera* root, *Ulmus davidiana* var. *japonica, Saururus chinensis, Gastrodia elata Blume,* and *Tripterygium wilfordii* (Seow et al., 2011).
- Solid tumors, in particular, desperately require new blood vessels for a sufficient supply of nutrients and oxygen, which are essential for growth over a few mm in size. In addition, new blood vessels enable cancer cells to invade adjacent tissue and to migrate throughout the body, which is known as metastasis. Therefore, suppression of such pathological angiogenesis has been one of the most promising approaches in prevention and treatment of cancer (Kim et al., 2014).
- Anti-angiogenic potential of test drugs is evaluated using vascularized chick embryo chorioallantoic membrane. Chick embryo chorioallantoic membrane (CAM) has become a widely used as evaluation methodology for the determination of both angiogenesis and anti-angiogenic activities of many drugs including herbal extracts (Seow et al., 2011).
- Accordingly for present study following medicinal plants are shortlisted for further literature survey so as to select a medicinal plant with anticancer potential for the present research study.

1. *Annona stenophyla*	2. *Eriobotrya japonica*
3. *Abrus precatorius* L.	4. *Citris karna* Raf.
5. *Annona cherimola* Mill.	6. *Borassus flabellifer* Linn.

3. RATIONALE / RESEARCH HYPOTHESES

- **Cancer cells are featured by** either fast multiplication or abnormal cell division and growth regulatory mechanisms. Normal host cell and cancer cell are distinguished by proliferation rate and cancer cells generally complement the biomolecules that are necessary for survival or proliferation, differentiation, cell death, and expression of many cell-type-specific functions. Improper regulation of controlled cell proliferation, distorted cell differentiation mechanism, deterioration of genetic and chromosomal organization, and disturbed programmed cell death pathways such as apoptosis could be responsible for the altered phenotype and formation of cancer. Hence, cancer or tumor formation could be controlled by controlling cell proliferation, differentiation, cell death and expression of many cell-type-specific functions. This mainly includes regulation of apoptic pathways and the genesis of cancer cell, expression of telomerase, tumor related blood vessels and interference with signal transduction (Andreef et al. 2012; Sharma and Allison 2015).
- **Kaempferol, luteolin, quercetin, genistein and curcumin, resveratrol, epigallocatechin gallate (EGCG), apigenin, lycopene, ursolic acid, isothyocynates, and perillyl alcohol** are a few examples of phytochemicals with anticancer potential. These anticancer bioactive compounds function by **diverse mechanisms** such as antioxidant properties, inhibition of cell cycle, induction of apoptosis, regulation of angiogenesis, inhibition of cyclooxygenase (COX)-2, signal transducers and activator of transcription 3 (STAT3) inhibition, and down-regulation of nuclear factor-kappaB (NF-κB), activator protein-1, and mitogen-activated protein kinases (MAPKs).and may affect more than one target within the cell (Thanekar et al., 2016; Kanda and Yokosuka 2015; Fritz et al. 2008; Yan et al. 2014).

- **Phytochemicals like polyphenolic** constitute one of the most prominent secondary plant metabolites, which are ubiquitous in the plant kingdom attracted the attention of researchers worldwide as nutraceutical in the prevention of several diseases. Many studies revealed that anti-cancer efficacy and action of synthetic or natural anticancer compound can be further enhanced by **combining them synergistically** with chemically similar or different compounds such as polyphenols. Polyphenols display many anticarcinogenic properties including their inhibitory effects on cancer cell proliferation, tumor growth, angiogenesis, metastasis, and apoptosis. In addition, they can also modulate immune system.
- The mixture containing **quercetin**, curcumin, green tea, cruciferex, and **resveratrol** significantly inhibited the growth of Fanconi anemia of head and neck squamous cell carcinoma by means of dose-dependent inhibition of cell proliferation response along with protection of normal cells against free radicals damage (Niedzwiecki et al., 2016).

4. OBJECTIVES OF RESEARCH

The objectives of the present study are:

1. Collection of potential ethno-medicinal plant with anticancer potential and selection of plant part containing bioactive phytoconstituents.
2. Phytochemical investigation and standardization of selected medicinal plant parts extracts/ fractions by qualitative and quantitative means of evaluation
3. Toxicity evaluation of plant extracts/ fractions (s).
4. Selection and procurement of bioactive phytochemical.
5. To study the anti-inflammatory, antiangiogenic and anticancer profile of plant extracts / fractions and bioactive phytochemical in the following ways and will be preliminarily consider for further pharmacological evaluations
    - Anti-inflammatory activity – *in vitro* mast cell, hyaluronidase & *in vivo* paw edema model.
    - Antiangiogenic activity – Chorioallantoic membrane (CAM) model
    - Anticancer activity – *In vivo* (using tumor inducer) and *in vitro* (cell line based) models.
6. To elucidate plausible action mechanism of extracts/ fraction and bioactive phytochemical.
7. To study possible effects of active extracts/ fractions in presence of bioactive phytochemical.
8. Acquisition of best possible understanding of antiangiogenic and anticancer mechanisms using *in silico* approaches such as Molecular docking, PASS, Molecular operating environment (MOE) software

5. PLAN OF WORK WITH DURATION OF STUDY

The experimental work will be divided into the following phases

Study phase		**Proposed work**
Phase I (6 month)	Preliminary study	➢ Procurement of plant material and bioactive phytochemical. ➢ Qualitative and quantitative analysis of plant extracts or fractions, and bioactive phytochemical. ➢ Toxicological evaluation plant extracts or fractions.
Phase II (6 month = total 12 months)	Model optimization	➢ Optimization of *in vitro* and *in vivo* models with dose / conc. of extracts/ fractions/ bioactive phytoconstituents ➢ Paw edema model for anti-inflammatory activity. ➢ To carry out *in vivo* anti-inflammatory model of extracts or fractions and bioactive phytochemical.
Phase III (6 month = total 18 months)	Primary screening of anti-angiogenic activity	➢ Selection and standardization of antiangiogenic model. ➢ To perform antiangiogenic activity using Chick embryo Chorioallantoic membrane (CAM) model of extracts or fractions and bioactive phytochemical.
Phase IV (6 month = total 24 months)	Elucidation of possible action mechanism	➢ To standardize and evaluate anticancer activity of active extracts/ fractions and bioactive phytochemical using *In vivo* animal model (using tumor inducer). ➢ To perform anticancer activity of active extracts/ fractions and bioactive phytochemical using *in vitro* cell line based models (Sulforhodamine B (SRB) assay)
Phase V (6 + 8 month = total 38 months)	(Extracts/fractions and bioactive phytochemical) Combined study	➢ To find out mechanism of action of active extract (s)/ fraction (s) and bioactive phytochemical with respect to mentioned hypotheses. ➢ To study possible effects of active extracts/ fractions in presence of bioactive phytochemical using *in vitro* cell line based models. ➢ Acquisition of best possible understanding of mechanism(s) by means of suitable methods (like *in silico* molecular docking)

Detail plan of work

Phase I

a) Procurement of plant material, bioactive phytochemical with standard drug:

- ✓ Plant material collection, identification and authentication.
- ✓ Processing of crude drug, pharmacognostic evaluation and extraction/fractionation of plant material.
- ✓ Bioactive phytochemical will be procured from authorized supplier with certificate of analysis.

b) Qualitative and quantitative evaluation (Standardization of extracts/fractions for content of bioactive phytoconstituents):

- ✓ Qualitative and quantitative evaluation of plant extracts or fractions and bioactive phytochemical can be done using pertinent spectrometric and chromatographic techniques.
- ✓ Determination of total polyphenolic, flavonoid. steroid, alkaloids, terpenoids, saponins using currently available methods.

c) Toxicological evaluation (Acute Oral toxicity study AOT):

- ✓ The AOT studies will be performed as per the OECD (Organization for Economic Co-operation and Development) Guidelines No. 425 to evaluate safety profile of plant extracts or fractions and bioactive phytochemical. AOT study will be helpful to determine the dose of plant extracts or fractions and bioactive phytochemical for screening study.

Phase II

a) Optimization of anti-inflammatory activity:

- ✓ Anti-inflammatory activity of extracts or fractions and bioactive phytochemical will be performed using one of *in vivo* anti-inflammatory model such as Paw edema model so as to know preliminary effects of extracts or fractions and bioactive phytochemical on inflammation **(Jung et al., 2009).**
- ✓ The following models are intended to be carried out to enumerate a few of them:
    1. Paw edema model
    2. Oxazolone-induced ear edema in mice
    3. Croton-oil ear edema in rats and mice
    4. Cotton wool granuloma
- ✓ The validation of selected model will be carried out in small set of animals and also animals will not be sacrificed at the end so animals will be subjected to succeeding further study.

b) Screening of anti-inflammatory activity on experimental animals:

- ✓ Animals used for standardization of anti-inflammatory model would be screened for anti-inflammatory actions prior and after treatment with the plant extracts or fractions, and bioactive phytochemical.

Phase III

a) Standardization of antiangiogenic model:

- ✓ Angiogenesis is a complex process of formation of new blood vessels during cell growth

cycle during normal body growth as well as abnormal cell multiplication like tumor or cancer generation.

- ✓ **Example:** Chick embryo Chorioallantoic membrane (CAM) model

b) Screening of antiangiogenic activity in Chick embryo Chorioallantoic membrane (CAM) model:

- ✓ Different doses of plant extracts or fractions and bioactive phytochemical would be screened for its antiangiogenic potential on the standardized Chick embryo Chorioallantoic membrane (CAM) model (**Yang et al., 2012; Pund et al., 2014; Li et al., 2013)**.
- ✓ The antiangiogenic effect will be recorded as 0 to 2. The score will be recorded independent three times in a blind manner. The average score will be calculated and the interpretation of anti-angiogenic activity will quantified as follows:

Phase IV

a) To evaluate anticancer/ antitumor activity using *In vivo* animal model (using tumor inducer):

- ✓ To standardize and evaluate anticancer activity of active extracts or fractions and bioactive phytochemical using *In vivo* animal model (such as Mousefibrosarcoma model).
- ✓ After primary screening plant extracts or fractions and bioactive phytochemical for anti-inflammatory and antiangiogenic activity, its effects on other tumor or cancer will be studied to investigate the probable mechanism of action as follows:

- ❖ ***In vivo*** **Evaluation of anticancer/ antitumor activity (Eg. Mouse fibrosarcoma**

model):

- ✓ A serially transplantable mouse fibrosarcoma developed in Swiss and will be used as a solid tumor model. The tumor induction and propagation was carried out as per protocols. Three diameters of the tumor will be measured along different planes, using vernier callipers, every day, after tumor transplantation. The average of the three diameters will used to monitor tumor growth. When the tumor will reach a palpable volume, 5–6 mm in diameter, mice were randomly grouped (6 animals each group) and dose regimen was commenced **(Thanekar et al., 2016; Yang et al., 2012)**.
- ✓ The influence of extracts or fractions and bioactive phytochemical on tumor growth can be studied by estimating various parameters as enlisted:

b) To evaluate anticancer/ antitumor activity of active extracts/ fractions and bioactive phytochemical using *in vitro* cell line based models

- ✓ Further cytotoxicity of active extracts or fractions and bioactive phytochemical will be evaluated using *in vitro* cell line based models such as Sulforhodamine B (SRB) assay.

❖ **Sulforhodamine B (SRB) assay:**

- ✓ The assay will be performed either at research center or it will be outsourced from Anti-Cancer Drug screening facility (ACDSF) at ACTREC, Tata Memorial Centre, Navi Mumbai.
- ✓ In short, the cell lines will grown in appropriate media and inoculated into 96 well microtiter plates. The extracts or fractions and bioactive phytochemical at fixed concentration 10 µg/ml, 20 µg/ml, 40 µg/ml, and 80 µg/ml will be added and incubated at standard conditions for 48 hours and assay will be terminated by the addition of cold TCA. Further Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid

will be added and excess dye will be removed by washing five times with 1 % acetic acid. The plates will be were air dried. Bound stain will be subsequently eluted with 10 mM trizma base, and the absorbance will read on an plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Suitable blanks and positive controls will be included **(Thanekar et al., 2016; Yang et al., 2012)**. Assays will be performed in triplicate and percentage of cell viability will calculated as follows.

Percent cell viable = [average absorbance of the test well/ average absorbance of the control] x 100

Phase V

a) To study possible effects of active extracts or fractions in presence of bioactive phytochemical using *in vitro* cell line based models.

- If selected bioactive phytochemical will produce tumor inhibitory effect (anticancer/antitumor) then it will be further explored for its anticancer activity.
- Plant extracts or fractions and bioactive phytochemical if found effective in the animal models, would be combined in various dose combinations for any synergistic action in the view of exploring selected plant extracts or fractions and bioactive phytochemical for the cancer as a novel approach/mechanism of disease management.

b) *In silco* confirmation of mechanism of actions:

- ✓ Confirmation of possible mechanisms using suitable *in silico* methods based on molecular docking **(Thanekar et al., 2016)**.

6. EXPECTED OUTCOME OF THE STUDY

- ✓ Exploration of a safe and effective extract (s)/ fraction (s)/ molecule or combination thereof which can act as a cytoprotective agent in the treatment of life threatening disorder like tumor/cancer.
- ✓ Gaining an insight into the plausible action mechanism for anticancer/antitumor potential of the test compound (extract (s) or fraction (s), bioactive phytochemical).
- ✓ Currently no effective cure for the cancer and existing drug therapies produce multiple side effects, this research proposal is endeavored to identify a potential phytochemical (polyphenols/ triterpenoids/ alkaloids) that would serve as a plausible anticancer/antitumor effect for betterment of patients' life in cancer like dreadful diseases/ pathologies.

7. FUTURE PLANS

1) Pharmacokinetic study:

After pharmacodynamic evaluation plant extract (s) or fraction (s), and bioactive phytochemical, further pharmacokinetic parameters would be evaluated in the view of their compatibility with respect to each other.

2) Receptor binding assay and biochemical analysis:

Further receptor binding assay and neurochemical analysis would be performed to reveal exact molecular mechanisms of action of individual plant phytochemical or bioactive phytochemical.

3) Exploration of standard drug for cancer/tumor treatment:

If selected standard drug will produce beneficial effect in the cancer/tumor disease, then it would be further evaluated for its pharmacokinetic profile, therapeutic dose, molecular mechanisms of action and possible side effects. It would be explored as a novel line of treatment for the management of different types of cancer/tumor.

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Place: Nanded
Date: 7 Aug. 2017

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